STABLE CELL LINES RESISTANT TO APOPTOSIS AND NUTRIENT STRESS AND METHODS OF MAKING SAME

BACKGROUND OF THE INVENTION

FIELD OF THE INVENTION

The invention pertains to the field of baculovirus expression. More particularly, the invention pertains to novel cell lines resistant to apoptosis and nutrient stresses.

DESCRIPTION OF RELATED ART

The baculovirus expression vector system is one of the most effective and widely used eukaryotic protein expression systems available. In the baculovirus expression system, foreign genes are inserted into the baculovirus genome and are typically expressed under the transcriptional control of one of the hyper-expressed baculovirus very late promoters, polyhedrin or p10. In addition to very high-level protein production, an additional advantage of the baculovirus expression system is the quality of post-translational processing of proteins. Foreign proteins expressed in baculovirus-infected insect cells are post-translationally processed in a manner very similar if not identical to that observed for proteins translated in other higher eukaryotes, particularly vertebrates. The baculovirus system is especially useful for production of secreted and membrane-bound proteins from higher eukaryotes since protein folding and processing in the baculovirus system is typically similar to other higher eukaryotes and usually results in biologically active proteins. However, some post-translational processes, such as trimming and modification of complex carbohydrates, differ from those observed in vertebrate cells.

Protein production in this expression system occurs in the context of a lethal infection. Thus, the physiological state of the host cell during infection may influence the level of recombinant protein translation, protein processing, and protein trafficking or secretion. Factors which influence host cell health or viability could influence protein production and quality, as well as the overall utility of this expression system. The health, growth, and propagation of host cells prior to infection by recombinant baculoviruses is

also an important practical concern for large scale production of proteins in the baculovirus system.

An integral component of baculovirus expression technology is the insect cell line in which recombinant proteins are expressed. Desirable characteristics for such cell lines include the capacity to scale cultures to high cell densities, cell growth in serum free medium, growth in suspension cultures, and high level protein expression. Two cell lines, Sf9 and Sf21, are routinely used since these cells grow to high density, express reasonably high levels of protein, adapt well to serum-free media formulations, and are easily adapted to large scale suspension cultures. Sf9 cells are a derivative of the Sf21 line.

It has been demonstrated that *Autographa californica* nucleopolyhedrovirus (AcMNPV) infection induces apoptosis in Sf21 cells if the AcMNPV p35 gene is inactivated or absent (Clem, R. J. *et al.*, 1991, Hershberger, P. A. *et al.*, 1994). Numerous subsequent studies further characterized the role and function of the P35 protein as a potent general suppressor of apoptosis (Rabizadeh, S. *et al.*, 1993, Sugimoto, A. *et al.*, 1994, Hay, B. A. *et al.*, 1994, Beidler, D. R. *et al.*, 1995, Martinou, I. *et al.*, 1995, Robertson, N. M. *et al.*, 1997).

The P35 protein inhibits programmed cell death by inhibiting cellular caspases, a family of cysteinyl proteases that are important components or effectors of the cell death pathway (Martin, S. J. and Green, D. R., 1995, Cryns, V. and Yuan, J., 1998). P35 has been shown to inhibit caspases belonging to groups I, II, and III and inhibition is believed to result from a multistep mechanism. P35 interacts directly with cellular caspases and is cleaved (at aspartate 87) by the caspase (Xue, D. and Horvitz, H. R., 1995, Bertin, J. *et* al., 1996, Bump, N. J. *et al.*, 1995, Fisher, A. J. *et al.*, 1999).

Cleavage of P35 is believed to result in a conformational change that is required for P35 inhibition of the caspase. Thus, P35 appears to represent an irreversible inhibitor of caspases since P35 fragments of 10 and 25 kDa remain associated with the caspase after cleavage at P35 residue Asp87 (Bump, N. J. *et al.*, 1995, Fisher, A. J. *et al.*, 1999).

However, P35 cleavage alone does not appear to be sufficient for inhibition of caspase activity since a single amino acid substitution mutation in P35 prevents the stable

association of P35 with the caspase (but not its cleavage), and P35 containing this mutation does not inhibit caspase-3 activity (Fisher, A. J., *et al.* 1999).

A previous study showed that the P35 protein expressed in stable cell lines was capable of inhibiting apoptosis induced by actinomycin D or by a mutant virus in which the p35 gene was deleted (Cartier, J. L., *et al.* 1994). In that study, stably transfected cells expressing p35 did not appear to have increased viability after infection, when compared with untransfected Sf21 cells. Resistance of those p35 expressing cells to nutrient stress was not examined, nor was the expression of secreted proteins from baculovirus expression vectors examined.

A mammalian oncogene, bcl-2, is normally expressed in the T and B-lymphocytes. It is believed that the product of this gene is a "survival gene" normally involved in enhancing cellular survival. Bcl-2 has been stably transfected into an immortalized cell line (Milner *et al.*, 1992). The stable transfectants were selected using G418.

Another study utilized these bcl-2 expressing cell lines to test for survival of these cells under apoptotic and high stress conditions (Singh *et al.*, 1996). The growth characteristics of the bcl-2 overexpressing cell lines were compared with control cells in stationary, suspension and serum-free cultures. In each of these cases, bcl-2 suppressed apoptosis and the cells were more robust than the control cells. This study concentrated on overexpressing bcl-2, a mammalian protein that is otherwise normally expressed in the cells. No viral genes encoding suppressors of apoptosis were incorporated into the cell lines. Bcl-2 is a suppressor of apoptosis that is expressed normally from mammalian cells and does not block apoptosis as broadly as certain viral suppressors of apoptosis, such as baculovirus P35, Cowpox CrmA or Vaccinia SPI-2. Thus, the use of viral suppressors of apoptosis may provide substantial advantages over cellular proteins. In the former study of bcl-2 expressing cells, only cell growth and responses to modified nutrient regimes were examined. They did not examine the effects of apoptotic suppression on foreign gene expression or on protein production.

Spodoptera frugiperda Sf9 cells provide desirable growth characteristics and are extensively used in both industrial and research applications. In general, recombinant protein expression in insect cells infected with baculovirus expression vectors is much

higher than in mammalian cells infected with vertebrate virus expression vectors. Sf9 cells, as well as most insect cell lines, are limited by their susceptibility to stress which results in the induction of apoptosis. Another widely used cell line derived from *Trichoplusia ni* (BTI-Tn5B1-4 or HighFive™ cells) provides greatly improved protein expression but is more difficult to grow in suspension cultures, and may not be suitable for some types of scale-up applications. Sf9 cells do not generate the same high levels of protein expression that can be achieved from Tn5B1-4 cells. Thus, neither of these cell lines provides the optimal combination of features for high-level protein production.

SUMMARY OF THE INVENTION

Cell lines that are commonly used for protein expression are engineered to include genes that encode suppressors of apoptosis (SA). Insect cell lines expressing these SA genes are resistant to apoptosis or programmed cell death, and have an increased capacity to express certain recombinant proteins. These cell lines also have increased resistance to many types of stress. Because some of the SA proteins inhibit apoptosis in a wide spectrum of organisms, these genes may be inserted into other plant or animal cell lines for a variety of purposes involving resistance to apoptosis or resistance to stress.

The cell lines of this invention have been stably transfected with a plasmid containing a gene for a suppressor of apoptosis. This gene is preferably the p35 gene from a baculovirus such as AcMNPV. The cell lines are resistant to both exposure to an inducer of apoptosis, such as actinomycin D, and nutrient deprivation. These cell lines also produced increased levels of recombinant proteins compared to the parental cell lines from which they were derived.

Cell lines expressing a suppressor of apoptosis, such as P35 or a tagged form of P35, are generated by a method of the invention. First, a gene for the suppressor of apoptosis is isolated. Then, a recombinant DNA expression vector is constructed to contain the suppressor of apoptosis gene. The recombinant DNA expression vector is capable of expressing the gene in a host. The plasmid is transfected into a host cell. The host cells are exposed to an inducer of apoptosis, such as actinomycin D. Those cells which survive exposure to the inducer of apoptosis are then cloned to create an apoptosis-

resistant cell line. In a preferred embodiment, the recombinant DNA expression vector is cotransfected with a second recombinant DNA expression vector containing a selectable marker, such as an antibiotic resistance gene. The second recombinant DNA expression vector is used to eliminate cells which have not been successfully transfected prior to selection with an inducer of apoptosis.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 shows insect cell expression constructs designed for expressing native and AcV5-tagged P35 proteins.

Fig. 2 shows secreted alkaline phosphatase (SEAP) levels measured from the supernatants of various cell lines infected with virus rAcMNPV-SEAP and grown in TNMFH medium containing 10% fetal bovine serum (FBS).

Fig. 3 shows beta-galactosidase levels measured from cellular extracts of various cell lines infected with virus AcMNPV-246 and grown in TNMFH medium containing 10% FBS.

Fig. 4 shows secreted alkaline phosphatase (SEAP) levels from the supernatants of cell lines infected with virus rAcMNPV-SEAP and grown in serum-free medium (Sf900-II).

Fig. 5 shows the detection and relative expression levels of P35 as established by Western blot analysis of $Sf9^{P35AcV5-1}$ and $Sf9^{P35AcV5-3}$ cell lines.

Fig. 6 shows an analysis of viability of various cell lines under conditions of nutrient stress.

DETAILED DESCRIPTION OF THE INVENTION

The present invention satisfies the need in the art for improved cell lines resistant to apoptosis. The baculovirus AcMNPV encodes a suppressor of apoptosis, P35, which is expressed early during the infection cycle and is known to inhibit apoptosis triggered by AcMNPV infection of Sf21 cells. Transient expression of P35 in Sf21 insect cells is also

known to prevent apoptosis. Since the baculovirus already contains a p35 gene, it is not intuitive to assume that, by adding this gene to an insect cell line, it would confer the observed benefits to the infected cells. Therefore, isolating a suppressor of apoptosis from a baculovirus and incorporating it into a cell line for this purpose is novel.

Sf9 cells were engineered to express P35, a baculovirus suppressor of apoptosis. The resulting P35-expressing cells were placed under selection using an inducer of apoptosis, such as actinomycin D. Cell lines cloned by this procedure expressed P35, were more highly resistant to nutrient stress, and yielded secreted recombinant proteins from baculovirus infections at levels similar to that achieved by Tn5B1-4 cells, currently one of the best cell lines available for recombinant protein production from baculovirus-infected cells.

To generate cell lines resistant to induction of apoptosis by stressful culture conditions, and to examine the effects of constitutive cellular P35 expression on protein production from baculovirus expression vectors, cell lines expressing AcMNPV P35 or an epitope-tagged P35 fusion protein were generated. Cell lines expressing P35 were first selected using a neomycin resistance gene and G418, then selected again in the presence of actinomycin D, an inducer of apoptosis in Sf9 cells.

Several clonal isolates were generated and examined for a) resistance to actinomycin D induced apoptosis and nutrient deprivation, b) growth in various media, and c) baculovirus expression of intracellular and secreted proteins. When compared with wild type Sf9 cells, two P35 expressing cell lines showed increased resistance to actinomycin D induced apoptosis, and a marked resistance to nutrient deprivation. When these cell lines were infected with a recombinant baculovirus expressing a secreted glycoprotein (Secreted Alkaline Phosphatase, SEAP), expression of the glycoprotein from these cells exceeded expression from wild type Sf9 cells, and was comparable to expression levels obtained from Tn5B1-4 cells. Proteins expressed in these cells may also be of higher quality, as protein processing may be, on average, more uniform or more complete than in the parental cells.

Cell Line Construction

The AcMNPV p35 gene (SEQ. ID. NO. 1; Ayres, M.D. *et al.*, 1994) was amplified from the AcMNPV genome and subcloned into an insect cell expression plasmid (p166BRNX) which contains an optimized baculovirus early promoter (Blissard, G. W. and Rohrmann, G. F., 1991, Monsma, S. A., Oomens, A. G. P. and Blissard, G. W., 1996). Although P35 may have more powerful inhibitory effects than other SAs, other suppressors of apoptosis, which include *Bombyx mori* P35 and inhibitors of apoptosis proteins (IAPs) from other baculoviruses, can be used similarly in the development of novel cell lines. Each of these SAs is derived from a baculovirus, and is not a part of the genome of the parental cell line. Two P35 insect cell expression plasmid constructs were generated. In one construct, the wild type AcMNPV P35 gene was cloned in its native form (amino acids 1-299). A second construct encoded the full length P35 ORF with an AcV5 epitope tag (Monsma, S. A. and Blissard, G. W., 1995) fused to the C-terminus of P35 (Fig. 1). The P35 amino acid sequence is shown in SEQ. ID. NO. 1 and SEQ. ID. NO. 2 (Ayres, M.D. *et al.*, 1994).

A plasmid (pl66BRNX-AcV5) for insect cell expression was constructed from the OpMNPV gp64 promoter region, a multiple cloning site, sequences encoding the AcV5 epitope, and polyA addition sequences from the OpMNPV gp64 gene (Fig. 1). The AcMNPV P35 ORF was PCR amplified and cloned into the EcoRI and XbaI sites. To construct this plasmid, a complementary oligonucleotide pair (SEQ. ID. NO. 3 and SEQ. ID. NO. 4) encoding a 5' Xba I site, the AcV5 epitope (SWKDASGWS; Monsma, S. A. and Blissard, G. W. 1995), two stop codons, and an XbaI compatible cohesive end at the 3' end was synthesized and cloned into plasmid vector p166BRNX digested with XbaI. The resulting plasmid, p166BRNX-AcV5, contains a 166 nucleotide OpMNPV GP64 early promoter region (Blissard, G. W. and Rohrmann, G. F., 1991) followed by a multiple cloning site containing BamHI, EcoRI, EagI, NotI, and XbaI sites, plus a polyadenylation signal from the OpMNPV gp64 gene. This plasmid was used to create the constructs below.

The P35 ORF was cloned in frame with the AcV5 epitope such that the expressed P35 protein contains a 2 amino acid linker and a 9 amino acid AcV5 epitope at the

C-terminus (Fig. 1). The AcMNPV p35 ORF (with no stop codon) was amplified by PCR from AcMNPV genomic DNA using two primers, p35upEcoRI (SEQ. ID. NO. 5, a primer that contained the sequences from the 5' end of the P35 ORF plus an EcoRI site at the 5' end) and p35lowXbaI-NO stop (SEQ. ID. NO. 6, a primer that contains sequences from the 3' end of the P35 ORF, but no stop codon, and a 3' XbaI site). The PCR product was cloned into EcoRI/XbaI digested plasmid p166BRNX-AcV5 to obtain plasmid p166-p35-AcV5. Plasmid pl66-p35-AcV5 contains the p35 ORF fused to a C-terminal AcV5 epitope tag, under the control of the OpMNPV gp64 early promoter (Fig. 1).

Construct p166-p35 includes a stop codon upstream of the AcV5 epitope and expresses a native P35 protein (Fig. 1). The p35 gene was amplified using primers p35upEcoRI (SEQ. ID. NO. 5) and p35lowXbaI-Stop (SEQ. ID. NO. 7) using the same strategy described above, except that the P35 stop codon was included in the 3' oligonucleotide and PCR product (Fig. 1). The resulting plasmid, p166-p35, expresses wild type P35. All of the constructs were confirmed by DNA sequencing.

Selection of Stably Transfected Cell Lines

Each P35 expression plasmid was cotransfected into Sf9 insect cells with a plasmid (pIE1-Neo) expressing Neomycin Phosphotransferase (NPT) and cells were selected using Geneticin disulfate (G418). Plasmid pIE1-Neo contains a bacterial NPT gene under the control of an AcMNPV IE1 promoter (Monsma, S. A., Oomens, A. G. P. and Blissard, G. W., 1996). Cells were also cotransfected with plasmid pl66-EGFP, which contains an enhanced green fluorescent protein gene under the transcriptional control of the OpMNPV gp64 early promoter (Chang, M.J., Kuzio, J. and Blissard, G. W., 1999). EGFP expression was used as a convenient visible marker for transgene expression in stably transfected cell lines. Other parental cell lines available for production of stably transfected cell lines include IPLB-Sf21, BTI-Tn5B1-4, BTI-MG-1, Tn368, Ld652Y, and BTI-EAA, any cell lines derived from the cell lines listed here, as well as any cell line susceptible to baculovirus infection. Those skilled in the art would appreciate that, in order to meet their unique expression needs, this method is applicable to cell lines not specifically listed. Examples of some of these cell lines are found in Granados, R.R. and Hashimoto, Y., 1989.

Sf9 cells were transfected using the $CaPO_4$ technique being known in the art, and herein incorporated by reference (Blissard, G. W. and Rohrmann, G. F., 1991). To generate cells expressing the epitope tagged P35 protein, Sf9 cells ($2x10^6$ cells) were transfected with plasmids pIE1-Neo (1 μg), p166-p35-AcV5 (5 μg), and p166-EGFP (1 μg). To generate cells expressing the native P35 protein, Sf9 cells were similarly transfected with plasmids pIE1-Neo (1 μg), p166-p35 (5 μg), and p166EGFP (1 μg). At 48 hours post transfection, G418 was added to the medium to a final concentration of 1 mg/ml and cells were incubated in G418 containing medium for three to four weeks. Under these conditions, only Sf9 cells that were stably transfected survived. Cells selected in G418 were also screened visually for EGFP fluorescence. Untransfected Sf9 cells were used as a negative control.

In order to select cells expressing higher levels of functional P35, the surviving transfected cells were placed in medium containing 0.1 μg/ml actinomycin D for 1 hour. Another example of an inducer of apoptosis that could be used to select cells resistant to apoptosis is UV irradiation. In mammalian cells, inducers of apoptosis such as tumor necrosis factor (TNF) could also be used. The selection using an inducer of apoptosis could alternatively be performed without cotransfection with a selectable marker such as an antibiotic resistance gene.

At the end of the one hour incubation in actinomycin D, the medium was replaced with fresh TNMFH medium (containing no actinomycin D) and cells were allowed to grow for three days. After three days, 85-90% of the cells treated with actinomycin D-containing medium died. Medium was replaced after 3 days and surviving single cells formed small colonies which were subsequently propagated and used to clone individual cell lines by limiting dilution. For limiting dilution cloning, cells were diluted into 96 well plates such that each well received only one cell on average. Each well was subsequently monitored to ensure that only a single colony was present. Monoclonal cell lines were selected and propagated. Cell lines expressing the native P35 protein were named $Sf9^{P35}$, and lines expressing the epitope tagged P35 protein were named $Sf9^{P35AcV5}$. Five stable cell lines expressing P35 were selected and named $Sf9^{P35-1}$, $Sf9^{P35-2}$, $Sf9^{P35-3}$, $Sf9^{P35-4}$, and $Sf9^{P35-5}$. Three cloned cell lines expressing the epitope tagged P35 protein were selected and named $Sf9^{P35AcV5-1}$, $Sf9^{P35AcV5-2}$, and $Sf9^{P35AcV5-3}$.

Growth Rates (in serum-containing medium)

Growth rates of selected cell lines were determined by plating $1x10^6$-$2x10^6$ cells in TNMFH medium supplemented with 10% fetal bovine serum in T-25 flasks, and monitoring cell growth at 24 hours intervals by a technique being known in the art, and herein incorporated by reference (Wang, P., Granados, R. R. and Shuler, M. L., 1992).

Growth curves were generated for cell lines $Sf9^{P35\text{-}1}$, $Sf9^{P35\text{-}3}$, $Sf9^{P35\text{-}4}$ and $Sf9^{P35\text{-}5}$, $Sf9^{P35AcV5\text{-}1}$, $Sf9^{P35AcV5\text{-}2}$, and $Sf9^{P35AcV5\text{-}3}$. To determine whether the growth rates of cell lines expressing P35 were affected by P35 expression or stable transfection, the growth curves were compared to the growth rate of Sf9 cells (Table 1). Under these conditions, the average doubling time for unmodified Sf9 cells was approximately 26 hours. Doubling times for stably transformed cell lines ranged from 23 to 39 hours, with most lines showing similar doubling times to Sf9 cells. Only cell lines $Sf9^{P35\text{-}3}$ and $Sf9^{P35\text{-}4}$ showed growth rates that were significantly extended in comparison to the parental Sf9 cell line.

Table 1 Growth rates of cell lines expressing P35

Cell line	Sf9	$Sf9^{P35\text{-}1}$	$Sf9^{P35\text{-}3}$	$Sf9^{P35\text{-}4}$	$Sf9^{P35\text{-}5}$	$Sf9^{P35Acv5\text{-}1}$	$Sf9^{P35Acv5\text{-}2}$	$Sf9^{P35Acv5\text{-}3}$
Doubling Time (h)	26	24	31	39	27	27	23	25

Baculovirus Expression and Reporter Gene Analysis

The novel cell lines may also preferably contain a recombinant DNA for expression of a recombinant protein. For example, two recombinant baculovirus constructs that express well characterized proteins were used to compare recombinant protein production in the cells stably transfected with P35 with production from standard insect cell lines. Virus rAcMNPV-SEAP encodes a truncated human placental alkaline phosphatase gene under the control of the AcMNPV polyhedrin promoter (Davis, T. R., Trotter, K. M., Granados, R. R. and Wood, H. A., 1992). This virus expresses secreted alkaline phosphatase (SEAP), a glycoprotein that is conveniently monitored by measuring SEAP activity from cell culture supernatants (Davis, T. R. *et al.,* 1993). A second virus, AcMNPV-246 (Wickham, T. J., Davis, T., Granados, R. R., Shuler, M. L. and Wood, H.

A., 1992), contains an *E. coli* Lac*Z* gene under the control of the AcMNPV polyhedrin promoter. Beta-galactosidase assays of infected cell lysates were used to measure the synthesis of this intracellular protein.

Secreted Alkaline Phosphatase (SEAP) Expression in Serum-Containing Media

To examine levels of SEAP expression from Sf9 or stably transfected cells expressing P35 in serum-containing medium, cells were plated in 24 well plates. For Sf9 cells and stably transfected cells expressing P35, $3x10^5$ cells/well were plated in each well of 24 well plates. Due to their larger size, Tn5B1-4 (HighFive™) cells were plated at a density of $1x10^5$ cells/well.

Cells were infected with rAcMNPV-SEAP at a multiplicity of infection (MOI) of 10 for 1 hour, then virus was removed and cells were placed in fresh medium. Supernatants were collected from infected cells at 2, 3, 4, 5, 6, 7, 8, or 9 days post infection (p.i.). Each time point in Fig. 2 represents SEAP accumulation from initiation of infection through the indicated time. For each time point, three separate replicate wells were infected and separate supernatants collected. SEAP activity was determined by a technique being known in the art, and herein incorporated by reference (Davis, T. R., Trotter, K. M., Granados, R. R. and Wood, H. A., 1992). Tn5B1-4 cells are substantially larger than Sf9 cells. Therefore, to accurately compare expression of SEAP from stably transfected Sf9 cells with that from Tn5B1-4 cells, SEAP activity was calculated on both a "per cell" and a "per biomass" basis and examined as: 1) international units (IU) /cell and 2) IU/mg cell protein. The total biomass of the Sf9 and Tn5BI-4 cells were compared using total protein content per cell to indicate biomass.

As estimated from Bradford protein assays, Tn5B1-4 cells contained approximately 0.353 mg protein per 10^6 cells, whereas $Sf9^{P35AcV5-1}$ cells (a representative cell line derived from Sf9 cells) contained approximately 0.208 mg protein per 10^6 cells. Thus, based on protein content, the biomass of an average Tn5B1-4 cell is almost 1.5 times that of an average Sf9 cell. Levels of SEAP were determined and are presented as International Units (IU) SEAP per mg cell protein (IU/mg cell protein) in Fig. 2. As expected, SEAP expression from Tn5BI-4 cells exceeded that from Sf9 cells, at most

times by approximately 2-3 fold when compared on a biomass basis (Fig. 2, 3-9 days post infection).

SEAP expression levels from all P35-expressing cell lines were higher than those from the parental Sf9 cells, and were generally comparable to SEAP levels from Tn5BI-4 cells, with the exception of measurements at 2-3 days post infection when SEAP levels in Tn5BI-4 cells exceeded all others (Fig. 2). SEAP accumulated to very high levels in one of the P35-expressing lines, $Sf9^{P35AcV5-1}$. SEAP levels in line $Sf9^{P35AcV5-1}$ were similar to those from Tn5B1-4 cells at 3-4 days post infection, but continued to accumulate to significantly higher levels (≥2-3 fold above Sf9 cell expression) at 5-6 days post infection. By 7-9 days post infection, SEAP levels from the $Sf9^{P35AcV5-1}$ line exceeded that from parental Sf9 cells by approximately 4 fold and were approximately 2 fold more than SEAP levels from Tn5B1-4 cells. High level protein production in $Sf9^{P35AcV5-1}$ cells appears to result from a prolonged infection cycle compared with the infection cycle of AcMNPV infected Sf9 cells. In typical baculovirus expression vector infection of Sf9 or Tn5B1-4 cells, foreign protein accumulation plateaus after approximately 120 hours post infection. In contrast, secreted proteins continued to accumulate until 216 hours post infection in both $Sf9^{P35AcV5-1}$ and $Sf9^{P35AcV5-3}$ cells.

B-Galactosidase Expression in Serum-Containing Media

Sf9 or stably transfected cells expressing P35 were plated at $3x10^5$ cells/well and Tn5B1-4 cells were plated at $1x10^5$ cells/well in 24 well plates to measure levels of B-galactosidase. Cells were infected with virus AcMNPV-246 (which contains an *E. coli* lacZ gene and expresses the B-galactosidase reporter protein) at an MOI of 10 as described above. Cells were collected at 2, 3, 4, 5, 6, 7, 8, or 9 days post infection for production of lysates (Fig. 3). Analysis of B-galactosidase activity was performed essentially by a technique being known in the art, and herein incorporated by reference (Wang, P., Granados, R. R. and Shuler, M. L., 1992). Lysates from each sample were prepared by placing cells in 100 µl PBS, then freezing and thawing the cells three times (10 min at -70°C, 10 min at 37°C) followed by centrifugation for 5 minutes at 12,000 x g to remove debris. Supernatants were decanted and 20 µl was used for each B-galactosidase reaction.

Each B-galactosidase reaction was incubated at 28°C and B-galactosidase activity was monitored by readings at OD420 nm and compared to a standard curve. International units (IU) of B-galactosidase activity were calculated as described earlier (Yu, Z., Podgwaite, J. D. and Wood, H. A., 1992). For each time point in Fig. 3, three infections were performed. Data collected at each time point represents B-galactosidase accumulation from initiation of infection through the indicated time. Because the cell types compared in this study differ in size and volume, expression levels of B-galactosidase were calculated as 1) international units (IU) /cell and 2) IU / mg cell protein.

Cell lines expressing AcMNPV P35 or tagged P35 were compared with Sf9 and Tn5Bl-4 cell lines. Generally, in infected P35-expressing cell lines, B-galactosidase levels were slightly higher than those observed in the infected control Sf9 cells (Fig. 3). One stably transfected P35 line, $Sf9^{P35AcV5-1}$, exhibited B-galactosidase expression levels that consistently exceeded those from Sf9 cells, with increased expression in this case ranging from approximately 2.3-2.7 fold from 4-8 days post infection. Thus, one P35-expressing cell line was superior to Sf9 cells for expression of B-galactosidase. However, expression from line $Sf9^{P35AcV5-1}$ did not exceed that in Tn5B1-4 cells under these conditions, as B-galactosidase expression in Tn5B1-4 cells was consistently higher than both Sf9 and the best stably transfected P35-expressing line (Fig. 3, Tn5B1-4 vs. $Sf9^{P35AcV5-1}$).

SEAP Expression in Serum-Free Media

Serum-free media formulations are routinely used for cell propagation and protein production from recombinant baculoviruses. For comparisons of Sf9 and P35 expressing cells in serum-free medium, Sf9, Tn5B1-4, and stably transfected P35 lines $Sf9^{P35AcV5-1}$ and $Sf9^{P35AcV5-3}$ were initially grown in TNMFH medium supplemented with 10% fetal bovine serum. These cells were subsequently adapted to serum-free medium (Sf900-II; Life Technologies, Inc., Rockville, MD). After adaptation to serum-free medium, these cells were passaged approximately eight times. Then, cells were infected with virus rAcMNPV-SEAP and SEAP assays performed on cell culture supernatants as described above. Supernatants were collected at 2, 3, 4, 5, 6, 7, 8, and 9 days post infection and SEAP levels determined.

When SEAP activity in serum free medium was examined, it was observed that, from 3-9 days post infection, accumulated SEAP levels in Tn5BI-4 cells were significantly higher (1.54 - 2.6 fold) than in Sf9 cells (Fig. 4). Also, from 5-9 days post infection, SEAP expression from stably transfected P35-expressing cells was significantly higher (1.7 - 2.2 fold) than in Sf9 cells (Fig. 4). Generally, SEAP expression levels from stably transfected P35 cells was similar to that from Tn5B1-4 cells, although one cell line ($Sf9^{P35AcV5-3}$) appeared to have slightly higher levels than that from Tn5BI-4 cells from 7-9 days post infection.

Western blot analysis

Increasing numbers of cells ($0.5x10^6$, $1x10^6$, and $2x10^6$ cells) from each cell line were examined on Western blots and relative P35 levels were quantified by fluorescence imaging. $Sf9^{P35AcV5-1}$ and $Sf9^{P35AcV5-3}$ cells were collected and washed twice with PBS. Proteins were denatured by heating to 100 °C for 5 minutes in Laemmli buffer and electrophoresed on a sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel. Proteins were transferred to Immobilon-P membrane (Millipore Corp., Bedford, MA) and the epitope-tagged P35 protein was detected using monoclonal antibody AcV5 (1:500 dilution) as a primary antibody, and a goat anti-Mouse IgG alkaline phosphatase conjugate as a secondary antibody (1:10,000 dilution). For semi-quantitative comparisons, an ECF substrate, Diethanolamine (Amersham Pharmacia Biotech, Piscataway, NJ), was used for protein immunodetection on a Storm laser scanning system (Molecular Dynamics, Inc.).

Fig. 5 shows the relative $P35^{AcV5}$ expression levels for $P35^{AcV5-1}$ and $P35^{AcV5-3}$ cells. The 1x column shows the relative expression levels from 0.5 x 10^6 cells, while the 4x column shows the relative expression levels from 2.0 x 10^6 cells. P35 fusion proteins were identified by incubation with MAb AcV5 and proteins were quantified by enhanced chemifluorescence.

From these comparisons, it was estimated that the AcV5-tagged P35 protein from line $Sf9^{P35AcV5-3}$ was present at levels approximately 1.5-1.7x above that detected from line $Sf9^{P35AcV5-1}$. Thus, the levels of P35 detected in the two cell lines do not differ substantially.

Gene copy number

Southern blot hybridization analysis was used to determine p35 gene copy number in stably transfected cell lines. A PCR amplified 908 bp DNA fragment containing the AcMNPV p35 gene open reading frame was labeled with either digoxigenin (DIG) using random primers (DIG High Prime Labeling and Detection Starter Kit 1, Boehringer Mannheim company) or with ^{32}P-dATP (DECAprime 11 Random Priming Kit, Ambion, Inc., Austin, TX), and used as a probe for high stringency hybridization analysis. For Southern blots, 20 μg DNA from each cell line ($Sf9^{P35AcV5-1}$ or $Sf9^{P35AcV5-3}$) was digested with EcoRI and XbaI, and electrophoresed and blotted onto positive charged nylon membrane (Micron Separations Inc.).

Either 0.1, 0.3, 0.5, 0.7 or 1.0 μg p166-p35-AcV5 plasmid DNA (digested with EcoRI and XbaI) was mixed with 20 μg Sf9 cell DNA (also digested with EcoRI and XbaI), electrophoresed and blotted to generate a standard curve for quantitative analysis. Increasing quantities of plasmid DNAs in each lane simulated increasing copy numbers of the p35 gene in the Sf9 genome. Hybridization data from these experiments was used to generate a standard curve for gene copy number. Two separate experiments were performed to compare a standard curve of p35 DNA to p35 DNA detected in lines $Sf9^{P35AcV5-1}$ and $Sf9^{P35\ AcV5-3}$.

The haploid Sf9 cell genome was estimated as approximately 5 x 10^8 base pairs, based on the approximated size of the *Bombyx mori* genome (Rasch, E. M., 1974, Gage, L. P., 1974). Estimates for gene copy number per haploid genome in $Sf9^{P35AcV5-1}$ and $Sf9^{P35AcV5-3}$ were derived from comparisons of hybridization signal strength between these cell lines and the standard curve reconstructed from plasmid DNA. Data were adjusted to the estimated size of the Sf9 genome. The data suggested that line $Sf9^{P35AcV5-1}$ contains approximately 12 copies of the p35 gene per haploid genome, while line $Sf9^{P35AcV5-3}$ contains approximately 2 copies of the p35 gene per haploid genome. Protein expression levels for these two cell lines do not correlate well with the relative number of copies of p35 in the genome. A possible explanation for this fact is that expression levels may be more dramatically affected by the site of integration rather than by the number of integration events.

Resistance of the Cell Lines to Actinomycin D and Nutrient Stress

The cells were examined for resistance to induction of apoptosis by actinomycin D, as well as survival under conditions of nutrient deprivation. Sf9 cells or stably transfected lines $Sf9^{P35AcV5-1}$ and $Sf9^{P35AcV5-3}$ were incubated in medium containing 0.1μg/ml actinomycin D for 1 hour, then placed in TNMFH medium. After three days, all Sf9 cells died, while both $Sf9^{P35AcV5-1}$ and $Sf9^{P35AcV5-3}$ cells survived. Thus, both P35-expressing cell lines exhibited resistance to actinomycin D.

Increasing doses of actinomycin D were tested to further examine the degree of resistance to this inducer of apoptosis. Sf9 or stably transfected cells expressing P35 were incubated in a range of concentrations of actinomycin D (.01 to 0.5 μg/ml) for one hour, then placed in TNMFH and scored for cell survival after 1-4 days (Table 2).

Apoptosis was induced in wild type Sf9 cells at actinomycin D concentrations between 0.05 μg/ml and 0.1 μg/ml (and higher) (Table 2A), whereas both P35-expressing cell lines ($Sf9^{P35AcV5-1}$ and $Sf9^{P35AcV5-3}$) were sensitive to actinomycin D only at higher concentrations between 0.25 μg/ml and 0.5 μg/ml (Table 2B and 2C). Resistance to actinomycin D as an inducer of apoptosis appears to be ≥ 2 fold, since survival of Sf9 cells was high (approximately 80-95%) at 0.075 μg/ml actinomycin D and was reduced to ≤ 5% at 0.1 μg/ml (Table 2A). In contrast, survival of both stably transfected lines was 100% at 0.25 μg/ml actinomycin D, and both were reduced to ≤ 10% at 0.5 μg/ml (Table 2B and 2C).

Table 2 Resistance to Induction of Apoptosis

(A) Sf9 Cell Survival (Percentage)

µg/ml Actinomycin D	0.01	0.025	0.05	0.075	0.1	0.25	0.5
Day 1	100	100	100	95	13.7	4.5	0
Day 2	100	100	100	90	8.6	3.6	0
Day 3	100	100	100	83	3.7	0	0
Day 4	100	100	100	83	0	0	0

(B) Sf9P35AcV5-1 Cell Survival (Percentage)

µg/ml Actinomycin D	0.01	0.025	0.05	0.075	0.1	0.25	0.5
Day 1	100	100	100	100	100	100	8.5
Day 2	100	100	100	100	100	100	5.7
Day 3	100	100	100	100	100	100	1.5
Day 4	100	100	100	100	100	100	0

(C) Sf9P35AcV5-3 Cell Survival (Percentage)

µg/ml Actinomycin D	0.01	0.025	0.05	0.075	0.1	0.25	0.5
Day 1	100	100	100	100	100	100	9.2
Day 2	100	100	100	100	100	100	8.5
Day 3	100	100	100	100	100	100	1.5
Day 4	100	100	100	100	100	100	0

To further examine resistance to apoptosis by P35 expressing cells, Sf9, $Sf9^{P35AcV5-1}$ and $Sf9^{P35AcV5-3}$ cells were plated at 2 x 10^6 cells/well in 6-well plates, then exposed to actinomycin D (0.1 µg/ml) for one hour, and incubated in fresh TNMFH

medium at 27°C for 24 hours. Cells were harvested and washed twice with PBS. Lysates were prepared by incubating cells in 200 µl lysis buffer (10mM Tris pH 7.5, 25 mM EDTA, 0.2% Triton X-100) for one hour at room temperature. The lysate was extracted once with phenol, once with phenol:chloroform (1:1), twice with chloroform, and then precipitated in two volumes of ethanol. Cellular DNA was resuspended in 30 µl water containing RNAse A (50 µg/ml). 10 µl of DNA from each cell treatment was electrophoresed on a 1.2% agarose gel in TBE buffer (not shown).

Examination of the DNA on ethidium bromide stained gels indicated that treatment of Sf9 cells with actinomycin D resulted in a DNA laddering effect typical of apoptosis. Degradation of the DNA into DNA ladders (compared to an untreated control) was apparent in the electrophoresed DNA from Sf9 cells. In contrast, the treated DNA extracted from both $Sf9^{P35AcV5-1}$ and $Sf9^{P35AcV5-3}$ cells showed little or no laddering or degradation. Thus, induction of apoptosis was not observed in $Sf9^{P35AcV5-1}$ and $Sf9^{P35AcV5-3}$ cells after exposure to 0.1µg/ml actinomycin D. These results are consistent with the cell survival studies shown in Table 2.

Since lines $Sf9^{P35AcV5-1}$ and $Sf9^{P35AcV5-3}$ were resistant to induction of apoptosis, these lines were also examined for resistance to nutrient stress by culturing cells in phosphate buffered saline (with no nutrients added) for extended periods. Sf9, $Sf9^{P35AcV5-1}$ and $Sf9^{P35AcV5-3}$ cells were plated in TNMFH medium. After a 2-hour attachment and equilibration period, TNMFH medium was removed and replaced with PBS (pH 7.1) and incubated at 27°C for 9 days. Cells were scored for viability at 24 hours intervals. The percentage of cells surviving at daily intervals and a comparison of cell survival are shown in Fig. 6. For example, when cells were examined at 20 hours post infection, $Sf9^{P35AcV5-3}$ cells were rounded with a large nucleus, dense cytoplasm, and little or no granularity of the cells. Essentially, these cells appeared healthy and intact. In contrast, Sf9 cells similarly incubated in PBS and examined at 20 hours post infection were mostly granular and shriveled in appearance, indicative of extensive cell lysis.

After 24 hours in PBS, viability of $Sf9^{P35AcV5-1}$ and $Sf9^{P35AcV5-3}$ cells was approximately 85% and 55% respectively. In contrast, viability of Sf9 cells was less than 5% at the same time point. Line $Sf9^{P35AcV5-3}$ exhibited the greatest degree of resistance to

nutrient stress as viability of $Sf9^{P35AcV5-3}$ cells remained above 40% after 4 days in PBS. Lines $Sf9^{P35AcV5-1}$ and $Sf9^{P35-3}$ also showed much greater resistance to nutrient stress than Sf9 cells, but in these lines cell viability dropped from ≥55% at 1 day, to around 15-20% after 3 days in PBS. No surviving Sf9 cells were observed after 4 days in PBS. Notably, some cells stably expressing P35 ($Sf9^{P35AcV5-1}$ and $Sf9^{P35AcV5-3}$) remained viable through 7-8 days in PBS. Thus, the stably transfected cell lines show an extraordinary resistance to nutrient deprivation when compared with unmodified Sf9 cells.

To examine the potential long-term effects of nutrient deprivation on $Sf9^{P35AcV5-1}$ and $Sf9^{P35AcV5-3}$ cells, these cells were placed in PBS buffer for 5 days as described above, then returned to TNMFH medium. In both cases, cells recovered and grew normally. When infected with the rAcMNPV-SEAP virus, expression levels of SEAP in the supernatant were similar to those from the same cell lines which had not been exposed to nutrient stress (not shown). Thus, exposure to extreme nutrient stress did not appear to affect the capacity of these cells to express recombinant proteins at high levels.

Conclusions

Foreign gene expression in insect cells has proven to be a tool of major importance in research, as well as industrial scale production of recombinant proteins. Sf9 cells are well suited for large scale culture applications, as they are known to express reasonably high levels of recombinant proteins and they adapt readily both to suspension culture and serum-free media preparations. However, Sf9 cells are somewhat fastidious, since they do not readily tolerate nutrient stress or high-density growth.

The invention demonstrates that by stably expressing a viral suppressor of apoptosis, such as the baculovirus P35 protein, in an insect cell line, such as Sf9, novel cell lines are generated and these cells are resistant to the induction of apoptosis, resistant to nutrient stress, and support substantially higher levels of protein expression than the parental cell line. The stable transfection of viral genes capable of suppressing apoptosis into a pre-existing cell line creates more hardy cells. The novel cell lines overcome the disadvantages of their parental cell line, Sf9. These cells express levels of recombinant proteins (secreted) at levels higher than that from the parental cell line (Sf9) and comparable to the most highly productive cell line available (Tn5B1-4). It is not entirely

clear how expression of P35 from the cell line results in these phenomena, since P35 is normally expressed from AcMNPV early in infection. It is possible that the accumulation of P35 in the cell prior to infection permits the infection to persist in the cells for longer periods or facilitates the maintenance of a more healthy cellular physiological state throughout infection. The presence of P35 in the cell before infection provides substantial benefits, including the expression of foreign genes.

Characterized cell lines derived from Sf9 cells and expressing the AcMNPV p35 gene are easily adapted to serum-free medium and grow readily in suspension cultures. Engineered suppressor of apoptosis genes, such as the AcMNPV p35 gene, as well as plasmids containing the suppressor of apoptosis gene(s) and antibiotic resistance genes, are useful for engineering other cell lines.

As an example, viral SA proteins could be engineered for expression in mammalian cells such as COS cells or NIH3T3 cells, for improved culture characteristics. The incorporation of viral SA proteins may affect production of valuable proteins in these cells or make them susceptible to additional pathogens. The replication of certain viruses in certain mammalian cell lines may be limited by the induction of apoptosis, which prevents productive infection and virus replication. By incorporating viral SA proteins into these cells, they may become useful for new applications, including protein expression in mammalian cells or the propagation or study of other viruses (for example, retroviral vectors).

The use of suppressors of apoptosis such as P35 improves cell lines for protein expression with baculovirus expression vectors. The P35-expressing cells have good market potential for industrial and/or pharmaceutical applications in which scaleup production of insect cells (prior to infection) is necessary, and high-level protein production is essential. In addition, Sf9 or other cell lines can be engineered with P35 or other suppressors of apoptosis. The technology is easy to apply to existing expression systems. It requires only the use of a different cell line for expression of foreign proteins. The invention permits increased flexibility in handling and scale-up of cell lines for many applications.

Accordingly, it is to be understood that the embodiments of the invention herein described are merely illustrative of the application of the principles of the invention. Reference herein to details of the illustrated embodiments is not intended to limit the scope of the claims, which themselves recite those features regarded as essential to the invention.

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